Sub-specific differentiation of intestinal spirochaete isolates by macrorestriction fragment profiling

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Macrorestriction fragment profile analysis by PFGE was used to distinguish intestinal spirochaetes, some of which were isolated from cases of swine dysentery and intestinal spirochaetosis in humans, pigs, mice, chickens and dogs. Macrorestriction fragment profiles using *Sma*I and *Sac*II restriction enzymes were produced and used in statistical analysis. This permitted the division of the isolates into two major clusters. One cluster contained isolates which were identified as *Serpulina pilosicoli* and the second cluster contained isolates identified as *Serpulina hyodysenteriae* by immunoblotting with species-specific mAbs. Both species contained sub-specific groups, although these rarely correlated with the source of the isolates. We conclude that PFGE is capable of sub-specific differentiation of intestinal spirochaetes, but that the current species contain a large variety of genotypes among which cross-species transmission may be feasible.

Keywords: intestinal spirochaete, pulsed-field gel electrophoresis, macrorestriction fragment profiling

INTRODUCTION

Spirochaetes involved in pathogenesis of intestinal disease are presently divided into two species, *Serpulina hyodysenteriae* and *Serpulina pilosicoli* (Trott *et al.*, 1996). *S. hyodysenteriae* is the aetiological agent of swine dysentery (Harris *et al.*, 1972). *S. pilosicoli*, which is weakly β -haemolytic has been implicated in 'intestinal spirochaetosis' where the presence of spirochaetes in the gut has been associated with diarrhoea in pigs (Lee *et al.*, 1993b), dogs (Duhamel *et al.*, 1995), birds (McLaren *et al.*, 1994) and humans (Lee & Hampson, 1994).

A number of techniques have been used in the analysis of intestinal spirochaete isolates. Multilocus enzyme electrophoresis (MLEE) of exocellular enzymes demonstrated that certain porcine and human isolates of *S. pilosicoli* were related (Lee & Hampson, 1994) whilst Coomassie-Blue-stained and [³⁵S]methionine-labelled SDS-polyacrylamide gels of whole-cell proteins showed great heterogeneity amongst human isolates (Barrett *et al.*, 1996). There are two further proposed species '*Serpulina intermedius*' (Lee *et al.*, 1993a) and '*Serpulina murdochii*' (Lee & Hampson, 1994) defined

on the basis of MLEE. The provisional designations of '*S. intermedius*' and '*S. murdochii*' were also applied to spirochaetes in the MLEE study of Stanton *et al.* (1996) although the strains of '*S. intermedius*' showed 98–99.9% sequence identity with the 16S rRNA genes of the reference isolates of *S. hyodysenteriae* (B78^T) and *Serpulina innocens* (B256^T). Analysis of unclassified isolates of intestinal spirochaetes from humans and dogs has shown some to be closely related although whether intestinal spirochaetosis is a zoonosis has yet to be clarified (Duhamel *et al.*, 1995; Koopman *et al.*, 1993). PFGE has been used for sub-species differentiation of *S. pilosicoli* (Atyeo *et al.*, 1996) and this study suggested the possibility of cross-species transmission, including zoonotic spread.

The main purpose of this investigation was to explore the genotypes of a large number of isolates of *S. pilosicoli* and *S. hyodysenteriae* to examine sub-species variation and look for evidence of cross-species transmission. We also sought correlations between genotypes and sources of the isolates.

METHODS

Bacterial isolates and culture conditions. Sixty-eight isolates of intestinal spirochaetes were obtained from a number of sources (Table 1). These 68 isolates included 52 from humans,

Abbreviations: MLEE, multilocus enzyme electrophoresis; UPGMA, unweighted-pair group method of arithmetic averages.

Table 1. Origin and source of the isolates used in this study

The numbers assigned to each isolate correspond with the numbers shown in Fig. 4. Numbers 1–58 are *S. pilosicoli* and 59–68 are *S. hyodysenteriae*.

No.	Isolate	Patient	Symptom	Source	Reference/supplier
1	1	Human: male aged 2	Diarrhoea, giardiasis	Oman	Barrett (1990)
2	26	Human: male aged 10	Diarrhoea, vomiting (1 d)	Oman	Barrett (1990)
3	3	Human: male aged 50	Abdominal pain	Oman	Barrett (1990)
4	4	Human: male aged 50	Giardiasis	Oman	Barrett (1990)
5	81/80	Human: male aged 69	Stroke/hemiplegia	France	Fournié-Amazouz <i>et al.</i> (1995)
6	12	Human: female aged 12	Abdominal pain (years)	Oman	Barrett (1990)
7	HRM3	Human: male aged 40	Constipation/diarrhoea	Italy	Sanna <i>et al.</i> (1984)
8	19	Human: female aged 5	Diarrhoea	Oman	Barrett (1990)
9	47	Human: female aged 40	Abdominal pain for 6 months	Oman	Barrett (1990)
10	39	Human: male aged 13	Abdominal pain	Oman	Barrett (1990)
11	40	Human: female aged 25	Flatulence	Oman	Barrett (1990)
12	43	Human: male aged 46	Abdominal pain	Oman	Barrett (1990)
13	3941	Human	Not known	Oman, no records kept	Barrett (1990)
14	64	Human: male aged 27	Abdominal pain/backache (3 years)	Oman	Barrett (1990)
15	6	Human: male aged 50	Abdominal pain/giardiasis	Oman	Barrett (1990)
16	17	Human: female aged 35	Not known	Oman	Barrett (1990)
17	13	Human: female aged 2	Diarrhoea for 2 weeks	Oman	Barrett (1990)
18	28/94	Human: male aged 55	Alcoholic with acute intoxication with ethylene glycol	France	Fournié-Amazouz <i>et al.</i> (1995)
19	HRM16	Human: male aged 22	Not known	Italy	Sanna <i>et al.</i> (1984)
20	29	Human: male aged 2	Abdominal pain (1 month)	Oman	Barrett (1990)
21	37	Human: female aged 30	Headaches (6 years)	Oman	Barrett (1990)
22	18	Human: female aged 26	Diarrhoea and vomiting	Oman	Barrett (1990)
23	20	Human: male aged 24	Diarrhoea/abdominal pain	Oman	Barrett (1990)
24	31	Human: female aged 10	Chronic diarrhoea	Oman	Barrett (1990)
25	25	Human: male aged 4	Diarrhoea for 2 weeks	Oman	Barrett (1990)
26	32	Human	Diarrhoea	UK, AIDS patient	S. Barrett
27	28	Human: female aged 45	Not known	Oman	Barrett (1990)
28	30	Human: male aged 60	Not known	Oman	Barrett (1990)
29	14	Human: female aged 20	Abdominal pain for 1 week	Oman	Barrett (1990)
30	PE90	Human: female aged 77	Stroke/hemiplegia	France	Fournié-Amazouz <i>et al.</i> (1995)
31	128/90	Human: male aged 69	Acute peritonitis	France	Fournié-Amazouz <i>et al.</i> (1995)
32	PWS/B	Pig	Post-weaning scour	UK	IAH, Compton*
33	10	Human: female aged 12	Acute lymphocytic leukaemia/abdominal pain/giardiasis	Oman	Barrett (1990)
34	A3888	Dog	Diarrhoea	Netherlands	Koopman (1992)
35	8	Human: female aged 12	Enuresis	Oman	Barrett (1990)
36	9	Human: female aged 4	Abdominal pain (1 year)	Oman	Barrett (1990)
37	M15183	Human: male aged 29	Diarrhoea/abdominal pain	Homosexual AIDS patient, UK	S. Barrett
38	A5660	Dog	Diarrhoea	Netherlands	Koopman (1992)
39	60	Human: male aged 3	Diarrhoea and vomiting	Oman	Barrett (1990)
40	RA87	Human: male aged 62	Severe arteriopathy	France	Fournié-Amazouz <i>et al.</i> (1995)

Table 1 (cont.)

No.	Isolate	Patient	Symptom	Source	Reference/supplier
41	36	Human: female aged 24	Abdominal pain/ <i>Shigella flexneri</i>	Oman	Barrett (1990)
42	Korlos	Human	Not known	Australian Aborigine	Lee & Hampson (1992)
43	23	Human: male aged 35	Abdominal pain	Oman	Barrett (1990)
44	A3077	Dog	Diarrhoea	Netherlands	Koopman (1990)
45	FT6	Human	Diarrhoea	AIDS patient, UK	S. Barrett
46	A5687	Dog	Diarrhoea	Netherlands	Koopman (1992)
47	Gel-2	Human: male aged 31	Diarrhoea	Homosexual AIDS patient, UK	S. Barrett
48	Gel	Human: male aged 31	Not known	Homosexual AIDS patient, UK	S. Barrett
49	38	Human: female aged 45	Abdominal pain/headache	Oman	Barrett (1990)
50	Matthews	Human: child	Diarrhoea	Australian Aborigine	Lee & Hampson (1992)
51	P43/6/78	Pig	Diarrhoea	UK	ATCC 51139
52	FT9	Human	Not known	UK	S. Barrett
53	FT7	Human	Not known	UK	S. Barrett
54	Wesley	Human: child	Diarrhoea	Australian Aborigine	Lee & Hampson (1994)
55	Jeremiah	Human: child	Diarrhoea	Australian Aborigine	Lee & Hampson (1994)
56	44	Human: male aged 29	Diarrhoea	Oman	Barrett (1990)
57	57	Human: male (child)	Not known	Oman	Barrett (1990)
58	35	Human: female aged 28	Fever, typhoid	Oman	Barrett (1990)
59	B78	Pig	Swine dysentery	USA	ATCC 27164
60	JWPM	Pig	Swine dysentery	Netherlands	Lemcke & Bew (1984)
61	B204	Pig	Swine dysentery	USA	B. van der Zeijst
62	A1 (ST.4)	Pig	Swine dysentery	Netherlands	B. van der Zeijst
63	11416	Chicken	Not known	Netherlands	IAH, Compton
64	P18A	Pig	Swine dysentery	UK	IAH, Compton
65	WT10/82	Mouse	Not known	Isolated from farm with swine dysentery, UK	IAH, Compton
66	C5	Pig	Diseased	Netherlands	ter Huurne (1993)
67	A4	Pig	Diseased	Netherlands	ter Huurne (1993)
68	AF6/80	Pig	Swine dysentery	UK	IAH, Compton

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10 from pigs, four from dogs, one from a chicken and one from a mouse. The isolates originated from the Muscat area of Oman ($n = 34$), Australia ($n = 4$), UK ($n = 12$), Italy ($n = 2$), France ($n = 5$), USA ($n = 2$) and The Netherlands ($n = 9$). Isolates 5, 18, 30, 31, 40 and 51 have previously been characterized as *S. pilosicoli* by Trott *et al.* (1997) and these isolates plus 48, 53 and 57 have been confirmed as *S. pilosicoli* using PCR to the 16S rRNA gene (K. A. L. De Smet, D. E. Worth & S. P. Barrett, personal communication). Isolates 59 and 61 were confirmed as *S. hyodysenteriae* by the use of species-specific PCR (Elder *et al.*, 1994). Spirochaetes were maintained in anaerobic conditions on Columbia agar base containing 5% (v/v) horse blood. Spirochaetes were cultured

anaerobically for DNA preparation in tryptone soya broth (Oxoid) containing 7.5% (v/v) horse serum (Oxoid), 0.25% (w/v) glucose and 0.05% (w/v) cysteine at 37 °C.

Preparation of high-molecular-mass genomic DNA. High-quality unsheared genomic DNA was prepared by embedding whole cells in chromosomal-grade agarose blocks then lysing the cells with detergent and proteinase K. Spirochaetes were harvested by centrifugation at 3000 *g* for 10 min. Forty milligrams (wet weight) of intestinal spirochaetes resuspended in 1 ml NET-100 were mixed with 1 ml molten low-melting-point chromosomal-grade agarose [0.9% (w/v) in NET-100; Bio-Rad] and dispensed into plugs (1 × 9 × 20 mm). Agarose

plugs were incubated (24 h, 37 °C) in lysis solution [6 mM Tris/HCl pH 7.6, 1 M NaCl, 100 mM EDTA, 0.5% (w/v) N-lauroyl sarcosine, 1 mg lysozyme ml⁻¹] followed by proteinase K treatment (48 h, 50 °C) in ESP [0.5 M EDTA pH 9.0 containing 1% (w/v) N-lauroyl sarcosine and 1.5 mg ml⁻¹ proteinase K (Boehringer)]. Agarose plugs were stored in ESP at 4 °C prior to TE (10 mM Tris/HCl pH 8.0, 1 mM EDTA) washing as described by Maslow *et al.* (1993).

Restriction digestion of DNA and PFGE. Rare-cutting restriction enzymes *Sma*I (5'CCCGGG) and *Sac*II (5'CCGCGG) were used to cleave the intestinal spirochaete genome. Portions (1 × 1 × 9 mm) of agarose plugs containing high-molecular-mass DNA were incubated for 15 min in restriction buffer (as supplied by the enzyme manufacturer) at 0–4 °C, the buffer was then replaced with buffer containing 40 units restriction enzyme and the 0–4 °C incubation repeated followed by 24 h incubation at the recommended temperature for the enzyme. The digested slivers were incubated at 50 °C for 20 min in ES [0.5 M EDTA pH 9.0 containing 1% (w/v) N-lauroyl sarcosine] followed by one wash in TE for 15 min and subjected to PFGE in 0.5 × TBE (1 × TBE is 90 mM Tris-borate and 1 mM EDTA pH 8.0) through 1% agarose (Bio-Rad) with a ramped pulse time of 10–60 seconds for 22 h then 70–90 s for 2 h using a Bio-Rad CHEF DR III apparatus at a temperature of 10 °C.

Gels were stained using 0.5 µg ethidium bromide ml⁻¹ for 30 min, de-stained in distilled water and visualized using a scanner (UVP Products).

Evaluation of pulsed-field gels. Band sizes were estimated using the Gelworks 1D software package (UVP products). At least two independent separations of the restriction fragments were performed to ensure reproducibility. Levels of relatedness of the isolates were determined by comprehensive pairwise comparisons of macrorestriction fragment sizes using the Dice coefficient (S_D). The S_D value is the ratio of twice the number of bands which each pair of restriction profiles has in common to the total number of fragments in each profile (Grothues & Tummler, 1991):

$$S_D = 2n_i / (n_A + n_B)$$

where n_A and n_B are the numbers of fragments in patterns A and B, respectively, and n_i is the number of common fragments. Mean values of S_D obtained were used in the UPGMA (unweighted-pair group method of arithmetic averages) software (Unistat version 4.0) to generate dendrograms of relatedness as described by Rainey *et al.* (1994).

SDS-PAGE. Separation of whole cell spirochaete proteins was carried out in the Mini-PROTEAN system (Bio-Rad) using a 12% (v/v) acrylamide separating gel and a 3% (v/v) stacking gel (Laemmli, 1970).

Immunoblotting. Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes (0.45 µm pore size, Bio-Rad) using a Mini-Transblot cell (Bio-Rad) (Towbin *et al.*, 1979). Two intestinal spirochaete-specific mAbs were used in this study: BJL/AC1, specific for a 29 kDa protein of *S. pilosicoli* (Lee & Hampson, 1995) and BJL/SH1, specific for a 30 kDa protein of *S. hyodysenteriae* (Lee & Hampson, 1996). Blots were visualized using anti-mouse IgG conjugated to horseradish peroxidase (Sigma) followed by development with 0.01% (v/v) hydrogen peroxide and 4-chloro-1-naphthol (25 µg ml⁻¹ in 10 mM Tris/HCl pH 7.4).

RESULTS

Identification of intestinal spirochaetes as *S. pilosicoli* or *S. hyodysenteriae*

The specificity of the two mAbs is illustrated in Fig. 1. Isolates 1–58 reacted with the *S. pilosicoli*-specific mAb. Isolates 59–68 were shown to react with species-specific mAb to *S. hyodysenteriae*.

Quantitative evaluation of macrorestriction fragment profiles

*Sma*I and *Sac*II were used to cleave the intestinal spirochaete genome into few fragments (Table 2). Other enzymes investigated (*Xba*I 5'TCTAGA, *Nae*I 5'GCCGGC) cut the genome into too many fragments to be useful in pairwise comparisons. Five isolates including *Brachyspira aalborgi* (Hovind-Hougen *et al.*, 1982) yielded too few fragments with *Sma*I and *Sac*II to be used in the statistical analyses and were therefore removed from the study. The size of the whole intestinal spirochaete genome was estimated to be approximately 2.6–3 Mb using PFGE following digestion with *Not*I which has a single restriction site (Zuerner & Stanton, 1994). For each isolate the approximate genome size was obtained by adding together the sizes of the resolved restriction fragments and was found to be 3 Mb. Fig. 2 shows the discriminatory value of the enzymes used in this study. Two isolates from a UK male homosexual (47 and 48) showed identical macrorestriction fragment profiles; otherwise substantial variation amongst the isolates was observed. Fig. 3 illustrates the discriminatory power of using two restriction enzymes to restrict the genomic DNA.

Fig. 1. SDS-PAGE immunoblot of representative isolates of *S. hyodysenteriae* and *S. pilosicoli* probed with the species-specific mAbs BJL/SH1 (a) and BJL/AC1 (b). Molecular mass standards are shown in kDa.

Table 2. Macrorestriction fragment sizes (kb) from *Sma*I and *Sac*II restriction digests of representative isolates from each of the clusters in Fig. 4

Cluster	Strain	<i>Sac</i> II	<i>Sma</i> I
1A	26	984, 585, 256, 202, 136, 62, 49	579, 382, 341, 329, 200, 115, 81, 52
1B	HRM3	964, 726, 426, 270, 214, 163, 112	693, 384, 365, 323, 123, 101, 64
1B	32	875, 542, 143, 88	951, 570, 447, 416, 374, 270, 99, 63
1B	PWS/B	884, 743, 452, 216, 185, 160, 102, 82	905, 868, 705, 320, 257, 177, 132, 102
1C	RA87	820, 569, 230, 194, 184, 141, 73, 57	909, 476, 300, 123, 94, 69, 56
1D	Gel-2	953, 482, 424, 230, 151, 138, 114, 56, 47	649, 373, 325, 129, 117, 108, 99
1E	44	582, 468, 433, 387, 205, 182, 152, 119	674, 441, 358, 296, 251, 220, 135, 104, 89, 66
2	B78	734, 508, 348, 309, 263, 209, 118, 54, 47, 40, 33	960, 252, 223, 83

Fig. 2. Variation in macrorestriction fragment profiles from *Sma*I-digested human isolates of *S. pilosicoli* isolated in Muscat, Oman. Lanes 1–9 correspond to isolates 1, 3, 4, 6, 9, 10, 12, 17 and 26 from Table 1. The sizes of co-migrating λ concatamers are shown in kbp on the left.

Division of the isolates into two major clusters

The levels of similarity of the profiles were determined by pairwise comparisons and quantified using S_D . The dendrogram in Fig. 4 shows two clusters at or below 1.8 Euclids. The range of macrorestriction fragment sizes of representative isolates in each of the clusters in Fig. 4 is shown in Table 2 for both *Sma*I and *Sac*II.

DISCUSSION

Application of PFGE to intestinal spirochaete isolates has confirmed the genomic heterogeneity of intestinal spirochaetes as a group and, more particularly, heterogeneity within *S. pilosicoli* (Atyeo *et al.*, 1996; Lee & Hampson, 1994). Cluster 1 contains isolates shown to be *S. pilosicoli* upon immunoblotting which are genotypically different, suggesting that specific division of *S.*

pilosicoli may be possible. This cluster was subdivided into five groups (A, B, C, D, E), all of which contain Omani human isolates. Few correlations could be made between the geographical origin of isolates and their genotype and it is noteworthy that human isolates collected in a limited area such as the city of Muscat in Oman were represented in almost every group within cluster 1.

Groups A and E of cluster 1 contained exclusively Omani isolates with group A including the Omani isolate 26 (2) which formed a separate branch in the study of Hookey *et al.* (1994). Group B contained the majority of the human isolates examined in this study. Groups C and D contained isolates from a wide range of sources confirming the sub-specific heterogeneity of *S. pilosicoli*.

The French bacteraemic isolates (5, 18, 30, 31, 40) (Fournié-Amazouz *et al.*, 1995) included in the study did not cluster together suggesting that the bacteraemic property cannot be correlated with genotype, although the number of isolates examined was small. Trott *et al.* (1997) also demonstrated that the French bacteraemic isolates did not cluster together using MLEE. The two isolates 47 and 48 were obtained at different times from the same male homosexual and had identical macrorestriction fragment profiles, demonstrating that the colonization with spirochaetes was stable and persistent. The persistence of colonization has also been shown in Aboriginal children (Lee & Hampson, 1992) and with *S. hyodysenteriae* in pigs (Neef *et al.*, 1994) and mice (Clark, 1984). The identical patterns obtained with the two isolates from a male homosexual suggest either that a long-term colonization occurred or that the patient was repeatedly infected with a single clone of spirochaetes. Other populations of spirochaetes may have been present in this patient and may not have been selected by the isolation procedure used. Three further isolates which were included from male homosexuals had different genotypes (45, 44, 51). Further studies would need to be carried out to establish whether male homosexuals were predisposed to infection with specific genotypes. No correlation could be found between disease manifestation and genotype in *S. pilosicoli*.

Fig. 3. PFGE illustrating the discriminatory power of the restriction enzymes *SacII* (lanes 2 and 3) and *SmaI* (lanes 4 and 5) on two isolates, JWPM (60) and B78 (59), used in this study.

Fig. 4. Dendrogram of intestinal spirochaete isolates generated by cluster analysis of macrorestriction fragment profiles using UPGMA.

In group C, the dog isolate A5687 (46) and the human isolate FT6 (45) showed similar profiles, diverging at a distance of 1.1 Euclids suggesting that cross-species transmission is feasible.

Cluster 2 contains 10 isolates confirmed as *S. hyodysenteriae* using the BJL/SH1 mAb. This group of isolates consisted predominately of pig isolates. Isolates 59 and 60 were sufficiently similar for discrimination to have occurred with only one of the restriction enzymes used in this study (*SacII*) (Fig. 3). Evidence was found that the profiles of the *S. hyodysenteriae* isolates showed some conserved fragments, suggesting that *S. hyodysenteriae* may be more conserved as a species than *S. pilosicoli*.

In conclusion, the heterogeneity of *S. pilosicoli* may reflect niche selection within a certain type of host, although there is no correlation between host specificity and genotype. Some evidence of cross-species transmission is suggested. Macrorestriction fragment profiling of intestinal spirochaetes has demonstrated sub-specific differentiation of isolates into heterogeneous groups, particularly in the case of *S. pilosicoli*.

ACKNOWLEDGEMENTS

We are grateful to G. Dettori, D. Hampson, I. Saint Girons and B. van der Zeijst for making their isolates available for study. We thank D. Hampson for the gift of mAbs. We thank M. Burrows for culturing isolates and P. Lambert for critical reading of the manuscript. S. J. R. was supported by a BBSRC studentship.

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Received 9 December 1996; revised 12 May 1997; accepted 23 May 1997.